STUDIES OF EARLY CLEAVAGE IN THE SURF CLAM, SPISULA SOLIDISSIMA, USING METHYLENE BLUE AND TOLUIDINE BLUE AS VITAL STAINS [1]

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In 1942 Iida described the behavior of certain particles stainable with neutral red in living eggs of a Japanese sea urchin. He found the particles to be one-half to one micron in diameter, to gather specifically into the regions of the asters during cleavage and to be distributed to the daughter cells by the mitotic spindle. In addition, he noted that the particles often moved very rapidly (several microns per second), but that their movement, especially after the establishment of the asters, was restricted to a direction radial to the centrosome. He also noted that particles would move both toward and away from the centrosome, and that of two neighboring particles, one might move and the other not. He thought that the particles were attached to astral rays and that the particle movement indicated intermittent growth (with periods of retraction) of the astral fibers. He compared both the form and activity of the aster to those of a radiolarian, centrosome representing the body of the radiolarian and astral fibers its filopodia.

Independently, Pasteels (1955, 1958), Pasteels and Mulnard (1957), Dalcq (1957) and Mulnard (1958) described particles stainable with basic dyes such as toluidine blue in living eggs of molluscs, annelids, echinoderms and ascidians, which at cleavage showed a behavior similar to, if not identical with, that first described by Iida (1942). Dalcq (1954) showed that rat eggs probably possess similar particles. The more recent work was considerably more complete and traced the origin and distribution of the particles from fertilization to later cleavage stages.

In addition, histochemical work indicated that acid phosphatase and acid mucopolysaccharides (the latter having been suggested by the fact that the particles stain metachromatically in the living egg) showed a distribution during cleavage similar to that shown by the particles in the living egg. Further, two types of particles were described, α-mitochondria not astrally located, appearing in the early fertilized egg, and β-mitochondria, appearing just prior to mitosis and showing the astral localization. Evidence was adduced to support the concept that the β-mitochondria were themselves not directly stainable, but became stained with the transfer of some substance to them from the α-mitochondria.

[1] This work was partially supported by grants from the American Cancer Society and from the National Science Foundation, and was completed in part while the author was at the Department of Anatomy, College of Medicine, University of Illinois.

[2] We wish to dedicate this paper to the memory of Dr. Victor Schechter who was our first teacher in Biology and who contributed much to present knowledge of the properties of the egg of *Spisula solidissima*.

The work to be reported below was started in 1956 without knowledge of these antecedent results and took as its starting point, observations on vital staining of invertebrate eggs reported in Worley and Worley (1943) and Worley (1944a, 1944b). In this work, methylene blue was used as a vital stain and a number of molluscan and other invertebrate eggs were used as material. Certain bodies, called "Golgi" bodies by the above workers, were seen in the cytoplasm after staining, and an elaborate cycle of growth, fractionation and re-growth of these bodies during cleavage was described. This cycle was thought to be correlated to the synthesis and elaboration of lipid and protein yolk.

Our interest in this work stemmed from observations made in the course of electron microscope studies of developing eggs of the surf clam, *Spisula solidissima* (Rebhun, 1956a, 1956b). Cytoplasmic bodies considered to fulfill the criteria for yolk nuclei were there described and it was hypothesized that "heavy" protein yolk was manufactured in them, although the evidence was weak and mostly by analogy to observations and conclusions concerning yolk nuclei found in the classical literature (see, *e.g.*, Wilson, 1925). The yolk nuclei appeared to us to resemble the "Golgi" bodies of the Worleys (Worley and Worley, 1943), especially in the lamellibranch *Mytilus,* whose egg resembles that of *Spisula* in many respects. This resemblance, coupled with the suggestions of yolk synthesis claimed for each body, led us to the hypothesis that the two descriptions were, indeed, concerned with only one object (Rebhun, 1956b). This hypothesis, which led to the work described below, was, however, negated at our next opportunity to study living, stained eggs; *i.e.*, yolk nuclei are visible as refractile bodies in living eggs but never stain with methylene blue. During the course of these studies, however, we found that the particles stainable with methylene blue undergo the localization changes during cleavage described for neutral red particles in sea urchins by Iida (1942).

After this paper was accepted for publication, the author became aware of two papers by Kojima (1959a, 1959b) in which particles stainable with toluidine blue, neutral red, etc., were shown to exhibit the same behavior as those described below. In addition, Kojima showed the particles to be involved in the process of cell division. This work is discussed in a later section.

The following paper is an expansion of results already presented in preliminary reports (Rebhun, 1957, 1958).

Materials and Methods

Oocytes were removed from ripe ovaries of the surf clam, *Spisula solidissima,* and washed following the procedure described in Allen (1953). They were then stained by either of two different procedures: that of Worley and Worley (1943), used throughout the major part of this work, and that of Pasteels (1955), used after we became aware of the latter's observations. In addition, some experiments were done with neutral red following Kojima (1959a).

In the method of Worley and Worley (1943), which we will call method I, eggs were stained by leaving them on a sea table at 19°–23° C. for ½ hour in a solution of 1 part methylene blue per 1,000,000 parts sea water, ½ hour in 1 part methylene blue per 500,000 parts sea water, 1 hour in 1 part methylene blue in 250,000 parts sea water, and finally, 1 hour in 1 part methylene blue in 125,000

parts sea water. Since there is variability in staining capacity in different batches of eggs (and with different lots of dye), the above schedule was somewhat varied in terms of staining time and final dye concentration used. However, a concentration of 1 part methylene blue per 125,000 parts of sea water was not exceeded. The eggs were then thoroughly washed by gentle centrifugation in fresh filtered sea water. The eggs of *Spisula* are very hardy (Schechter, 1941) and this long sojourn in dye solution does not appear to cause injury to the eggs. This may be judged by the fact that they cleave with the same frequency and at the same rate as control unstained eggs from the same batch, and that they develop for the same length of time on the sea table (namely, about three weeks), reaching the same final stage.

Eggs stained as above will be called "lightly" stained eggs. Their cytoplasm contains many small (about ¼ to ½ micron) particles, each heavily stained. If eggs are left in the highest concentrations of dye for 1 to 2 hours longer than described above, the particles appear to increase in size (but not number) until they are 1 to 2 microns in diameter, without, however, showing any dilution in color intensity. Such eggs will be called "heavily" stained eggs. Azure A and Azure B were also used in this procedure and yielded similar results, except that developmental anomalies were more frequent with the Azures than in unstained controls. These dyes give more intense staining, and concentrations of 1 part dye per 250,000 parts sea water were not exceeded with them.

The above method is obviously suited only to unfertilized eggs because of the extensive staining periods, and, therefore, for many purposes, the method of Pasteels (1955) was used. We will call this method II. Solutions of toluidine blue at concentrations of about 1 part dye per 100,000 parts sea water were used (this is about one-fifth the concentration used by Pasteels, 1955), and the eggs were allowed to remain in such solutions for from 2 to 5 minutes. The eggs were then removed and washed thoroughly by repeated centrifugation with fresh filtered sea water in a small hand centrifuge. Concentrations of toluidine blue of 1 or more parts dye in 50,000 parts sea water will stain the thin jelly coat and the vitelline membrane, reduce the fertilization percentage, often induce some of the eggs to develop parthenogenetically, and cause the appearance of angular and elongate particles in the cytoplasm which may be crystalline aggregates of dye. At the lower concentration of 1 part toluidine blue in 100,000 parts sea water, eggs stained when unfertilized will subsequently show no difference in fertilization percentage, cleavage rate and length of developmental period on the sea table, when compared to "sibling" controls. Similarly, eggs stained with the lower dye concentration at any time during the mitotic cycle compare closely with controls in the above characteristics.

In some experiments "mitochondrial" techniques were used. The Nadi reaction was used according to Ries (1937). Zinc-free Janus Green B (kindly given to us by Dr. D. P. Costello) solutions at a concentration of 1 part dye in 50,000 parts sea water were used to stain eggs at various times in the mitotic cycle. Finally, the NBT method of Nachlas *et al.* (1957) was tried. Although this latter technique did not yield consistent results as far as appearance of eggs from different batches is concerned, it did induce interesting anomalies in cleavage which will be reported at a later date.

Photomicrographs were taken through a Leitz Ortholux microscope with Microibso attachment and Leica camera using Kodak Microfile film and D-19 developer. Over 2000 feet of time-lapse movies were made with Plus X and Tri-X 16 mm. movie film of fertilized, stained eggs at many periods from fertilization to fifth cleavage. The movie equipment used consisted of a Bolex movie camera and Samenco movie control box. The camera was supported above the microscope and was driven at rates of 1 frame per second, or 1 frame per 2 seconds by a relay actuated by the control. The light is on constantly with the Samenco control for speeds as rapid as the above. Films were analyzed visually by timing events from a screen.

In some experiments, stained eggs were centrifuged in a small high speed electric centrifuge, the speed of which was altered with a Variac. The centrifuge was calibrated with a stroboscopic lamp (Strobotac). Eggs were centrifuged against isotonic sucrose (0.95 molal) in Pyrex tubes (Kopac, 1955). Complete stratification could be obtained with 1½ to 4 minute spins at 8000 g (depending on the batch of eggs), but various regimes were tried on a given sample of eggs, *e.g.*, 1 minute at 2000 g, 1 minute at 5000 g, and 2 minutes at 8000 g. This was considered to lessen the chance of accidental trapping of particles, *e.g.*, above the nucleus. A more complete description of centrifugation techniques will be reported in the observations section.

Observations with several phase systems and the Baker interference microscope (using both shearing and double focus systems) were made on normal eggs at various times in the cleavage cycle. To help alleviate the halo and scattering effects due to refractile granules in the cytoplasm, Dr. Keith Ross and the author attempted to find an immersion medium (Ross, 1954) which would allow cleavage to proceed normally and which would match the average refractive index of the egg cytoplasm. We used bovine serum albumin, Armour fraction V in sea water, gum arabic in sea water, and a high molecular weight polyglucose, "Ficol," dissolved in sea water. Concentrated solutions were made and dialysed against sea water in a refrigerator overnight. Eggs were immersed in this medium (or dilutions thereof) on slides and were then observed.

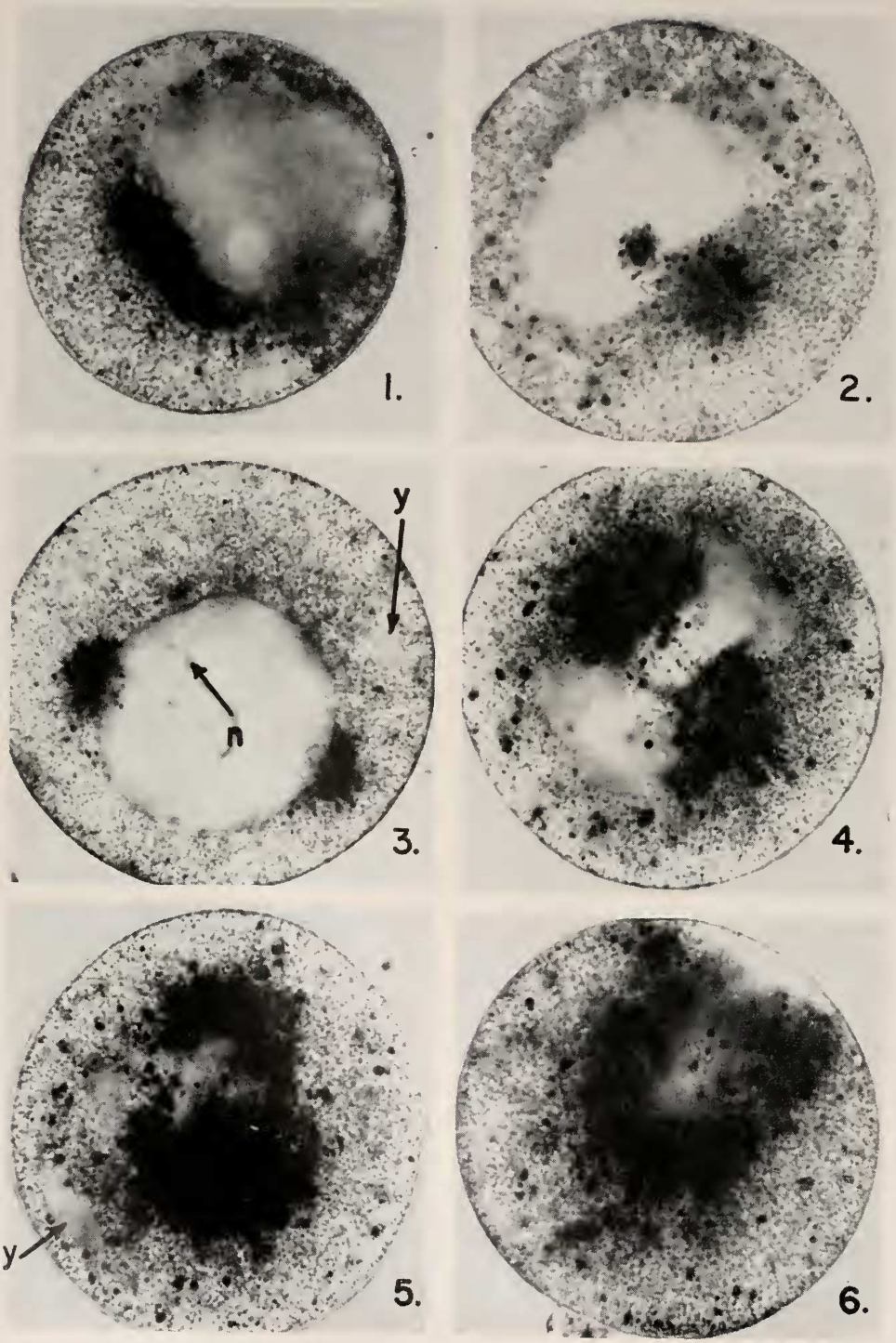
A complete report of our electron microscopic techniques will be made at a later date, although some of the results already obtained will be mentioned below.

Observations

I. *Unfertilized eggs:*

a. *Method I*

As mentioned above, small stained particles appear in eggs after several hours in solutions of methylene blue. With increased staining time the particles grow from about ¼ to ½ micron up to 1 to 2 microns in diameter. This does not appear to cause a decrease in the light absorption of the bound dye as might be expected if the dye were being diluted in a swelling vesicle. The particles appear dark whether the egg is lightly or heavily stained, and in fact, the larger particles appear darker. When eggs are removed from the staining solutions, by repeated washing in sea water, the particles do not increase further in size. Sim-



FIGURES 1–6.

ilarly, fertilization of eggs in staining solutions appears to prevent further increase in the size of the particles even though the dye is still present in the sea water.

Eggs left in staining solutions for 6 to 8 hours begin to show the formation of compound bodies with several particles aggregated into rows or around clear areas ("vacuoles") of cytoplasm 3 to 5 microns in diameter. Occasionally, very large spherical bodies up to 10 microns or more may be seen, which stain heavily with dye. We believe these compound bodies to be artifacts produced in the continued presence of dye. Yolk nuclei are visible in unstained and stained eggs (*e.g.*, see Figure 3), but, as mentioned in the introduction, never take up dye although a stained particle or two may appear within the interior of the spherical type of yolk nucleus (Rebhun, 1956b).

b. *Method II*

After short-period staining with toluidine blue, very small particles (¼ to ½ micron in diameter) appear in the egg and are uniformly distributed throughout the cytoplasm in most eggs. However, in some eggs, there appears to be a greater localization in the cytoplasmic region nearer to the nucleus. The particles take a reddish hue; *i.e.*, they stain metachromatically. The outermost layer of cytoplasm (about 1 to 2 microns thick) just beneath the vitelline membrane contains a set of particles which are somewhat elongated and are about 1 micron in over-all length although much variability in size exists. With the staining regime of method II, these cortical granules stain light blue, that is, non-metachromatically (in methylene blue solutions the granules stain light green). The two types of stained particles are thus easily distinguished by size, color, and location.

II. *Fertilized eggs:*

a. *General*

Particles in lightly stained and heavily stained eggs (method I) (taken out of dye solutions before compound bodies formed) and those stained by method II showed approximately the same behavior during fertilization and cleavage. However, until the stage of syngamy there is much more "diffuse" coloration (metachromatic) with method II than method I, which tends to obscure the events in the early stages. Most of the photomicrographs and movies, therefore, were taken

FIGURE 1. A lightly centrifuged egg with methylene blue stained particles accumulating just centrifugal to the nucleus.

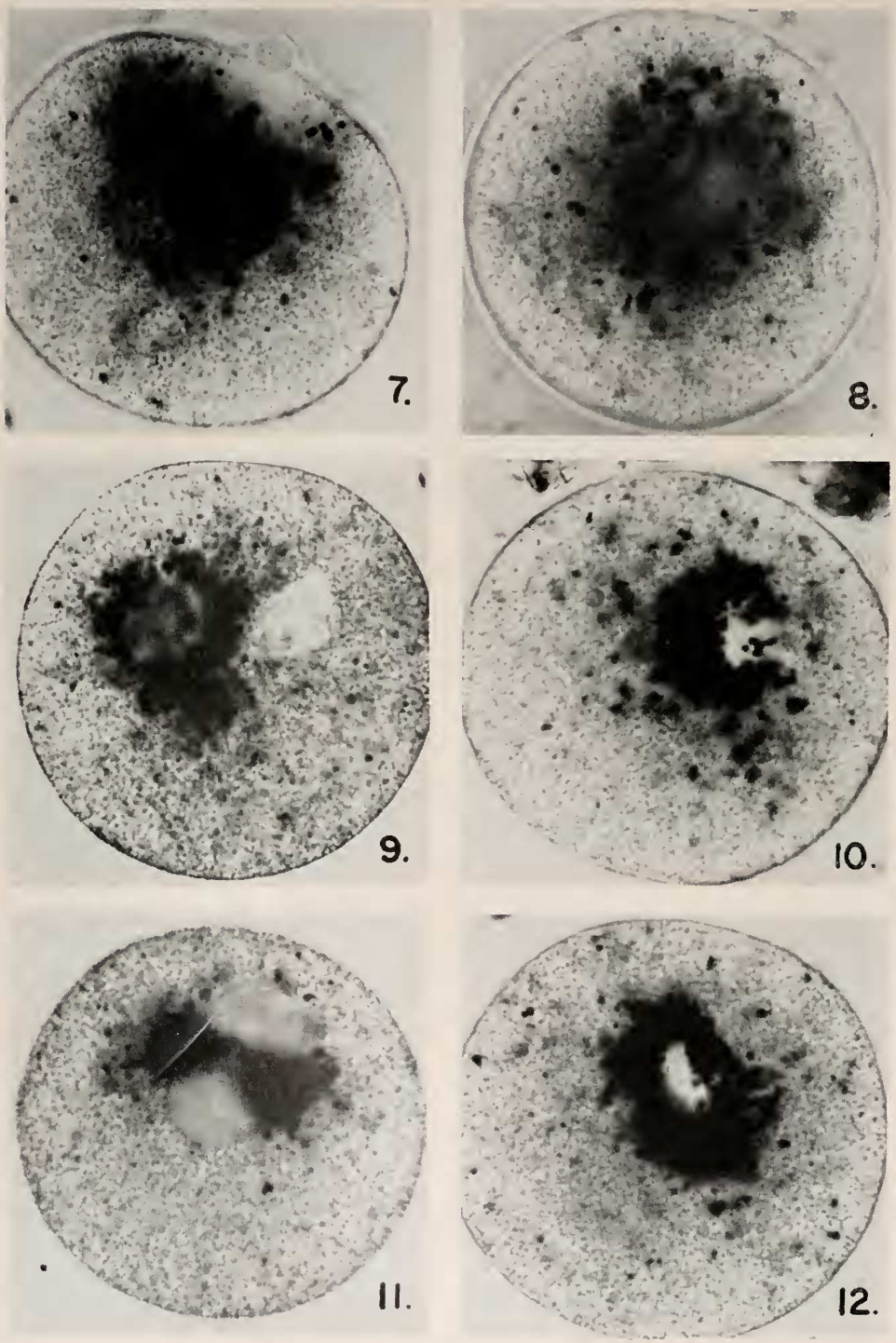
FIGURE 2. A parthogenetically activated, centrifuged egg. The aster is expanding into the nucleus.

FIGURE 3. A fertilized methylene blue stained egg. Many of the particles are gathered about the egg centrosomes, and astral rays are just beginning to indent the nuclear surface. About 8 minutes after fertilization. N = nucleolus, y = yolk nucleus.

FIGURE 4. At about 13 minutes after fertilization the astral rays have bridged the nucleoplasm and the spindle is being established. Note the masses of nucleoplasm at either side of the spindle.

FIGURE 5. At about 18 minutes the spindle is complete and begins its peripheral migration. The "excess" nucleoplasm has become intermingled with the cytoplasm.

FIGURE 6. At about 25 minutes the peripheral position of the spindle is established. Note the clear peripheral area. This is reminiscent of that seen later for first and second cleavage spindles.



FIGURES 7–12.

with heavily stained eggs which lack this "diffuse" coloration. Unfortunately, these eggs appear to be more sensitive to the light (or heat) of the microscope lamp than lightly stained eggs or unstained controls (despite heat filters and water cells) and movies of them cannot usually be taken for longer than 15 minutes. However, heavily stained eggs develop perfectly well on the sea table so that slides can be made as needed from a batch fertilized at one time.

b. *Formation of the first polar body spindle*

At about 6 to 8 minutes after fertilization, asters may be seen in the cytoplasm of the eggs, adjacent to the nuclear membrane: these may appear facing each other across the germinal vesicle or may be nearer to each other along the nuclear surface. A minute or two later the nuclear surface appears to soften, the nucleus actually enlarging (see Figure 3). The asters also begin to enlarge and the rod-like astral fibers facing the nucleus appear to push their way into the nucleus through its softened surface (Fig. 4). They elongate at the rate of about 1 micron every 2 to 3 seconds, occasionally pushing refractile (not necessarily stained) granules ahead of them from the cytoplasm into the nucleus. The cytoplasmic granules accompanying the nuclear invasion by the asters form a bridge of cytoplasmic material between the two asters (Fig. 4). In some eggs the stained particles begin to show a specific movement toward the astral centers. This is developed to a variable extent in different eggs at this time but is often quite definite. As the spindle (first polar body spindle) forms between the asters, the particles migrate more and more into the astral regions. The spindle elongates after its formation and at about 15 minutes after fertilization begins to move from the center of the egg toward the surface where the first polar body is given off (Figs. 5, 6).

When the spindle first forms, two large clear areas of nucleoplasm can be seen on either side of it (Fig. 4). They may be unequal in size, depending on the positions of the asters just before the nuclear membrane disappears. When the spindle begins its peripheral migration this nucleoplasm becomes increasingly mingled with the cytoplasm and finally can no longer be distinguished as a separate

FIGURE 7. The first polar body is formed by 31 minutes and the egg still has its oblate spheroid shape. Most of the particles are aggregated about the spindle or are, at least, in the animal hemisphere.

FIGURE 8. An optical section of the first polar body spindle perpendicular to its axis. The particles are distributed relatively uniformly. Note that many of them are aggregated into larger masses. Although difficult to photograph, these masses appear to be aggregates of smaller particles when studied visually.

FIGURE 9. The male pronucleus has not completely rounded out yet but is near the female pronucleus. About 50 minutes after fertilization.

FIGURE 10. A view along the line connecting the pronuclear centers reveals the particles lying approximately in a plane tangent to the two pronuclei at their point of contact (see Figure 11). The group of particles may be arranged in a crescent as here, or may form a complete ring. Fifty-three minutes after fertilization.

FIGURE 11. Looking at the pronuclei in a direction perpendicular to that of Figure 10 reveals the as yet intact pronuclei and the ring of particles in the plane tangent to the pronuclei. Same time as Figure 11.

FIGURE 12. The ring of particles begins to separate into two groups which will outline the asters of first cleavage. This process begins between 53 and 54 minutes after fertilization at 21° C.

entity (Fig. 5). Although some of the nuclear material may contribute to the spindle, the majority of it can be seen to mix with the cytoplasm.

From the time the asters first become visible, some of the stained particles migrate toward them (always excluding a zone about 4 to 5 microns in diameter centered on the centriole). This migration increases in intensity so that by the time the first polar body is given off at about 30 minutes, most of the particles are at least in the animal hemisphere, if not directly applied to the central aster and spindle (Figs. 6, 7). Some, but not all, of these particles move with great rapidity (several microns per second) and may appear to shoot quickly into the aster in time-lapse movies.

Many of the particles aggregate into what appear to be grape-like masses oriented radially along the astral rays (Figs. 6–8). This does not appear to be merely due to mechanical jostling since such aggregates may be seen in areas devoid of astral rays. When the spindle is first formed the stained particles outline each aster about equally. However, after the peripheral location of the spindle is established, the particles about the peripheral aster migrate to the sides of the spindle so that it is now outlined by a cup-shaped set of particles (Figs. 5 and 6). An optical section of the spindle and particles, perpendicular to the axis of the spindle, appears in Figure 8.

c. *Formation of first and second polar bodies*

At about 27 minutes after fertilization the egg begins to elongate in a direction at right angles to the animal-vegetal axis so as to become an approximate oblate spheroid (Fig. 7). The first polar body is given off at about 30 minutes (at 21° C.) and is accompanied by what appears to be a shortening, and a movement, of the spindle partly into the polar body (see Conklin, 1902, for a similar phenomenon in *Crepidula*). One or two stained particles may move into the polar body. The egg then rounds up.

Ten minutes later the second polar body is formed after a change in egg shape similar to that which occurred in first polar body formation. Throughout this period more and more of the laggard stained particles have been moving towards the spindle area and by now very few can be found elsewhere in the egg. A tight, organized mass of these particles exists around the second polar body spindle and central aster, and within this mass one can see, by careful focussing, the groups of particles forming grape-like masses.

Again, it must be emphasized that these observations can be made in eggs stained by method I or method II, although they are easier to make using method I because of the "diffuse" stain with method II.

d. *Formation of the pronuclei and their subsequent migrations*

A few minutes after second polar body formation the female pronucleus can be seen with the attendant mass of stained particles. These may completely surround it or may be gathered at one pole (pointing essentially toward the egg center). Whatever the initial distribution, however, ultimately the particles gather at the central pole of the female pronucleus.

The earliest stages of male pronucleus formation have not been seen but at

about the time that the female pronucleus becomes visible, the male pronucleus is already present and at this time appears as a clear body smaller than the female pronucleus and shaped like a short, very thick exclamation mark pointing toward the egg interior (Fig. 9). At the "period" of the exclamation mark is the center of the sperm aster which soon radiates throughout the cell. The male pronucleus increases in size and becomes spherical and about the same size as the female pronucleus.

The pronuclei have moved toward each other and by now the stained particles form a ring or crescent on the side of the female pronucleus facing the male pronucleus. The two pronuclei soon come into contact. A line drawn through their centers runs parallel to the animal-vegetal axis in the most frequent cases. The ring of particles is bisected by a plane which is tangent to the pronuclei (and thus perpendicular to the animal-vegetal axis). For views along and perpendicular to the animal-vegetal axis, see Figures 10 and 11, respectively.

It will be noticed in the above description that the particles stayed with the female pronucleus and did not move toward the male aster. This has been seen invariably in these eggs with both staining methods. However, in polyspermic eggs some particles may be seen to move into the sperm asters, usually with great rapidity (about a micron per second or so), although the bulk of particles remain with the female pronucleus.

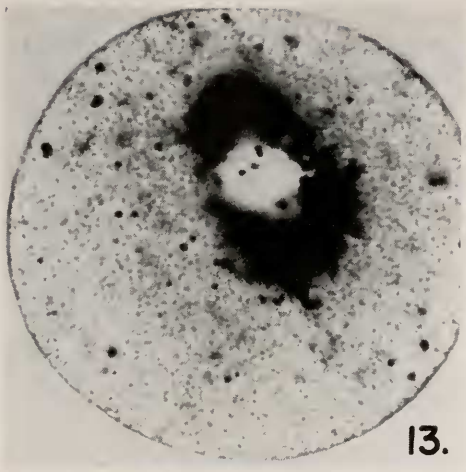
Preliminary studies have been made on parthenogenetic eggs stained with methylene blue and stimulated with KCl (see Allen, 1953). The events up to second polar body formation appear to be identical to those in the normal fertilized egg. However, a female pronucleus does not form in such eggs, although several small clear vesicles do, and, although observation of their relation to the particles is difficult, it is likely that the particles associate randomly with the vesicles. It would appear that the vesicles are similar to the karyomeres which form just prior to blastomere nucleus formation in first cleavage (see below). Although vesicle and chromosome counts were not made, it appears likely that each chromosome forms a karyomere and that, in parthenogenetic eggs, fusion of the karyomere to form a nucleus (pronucleus) does not occur.

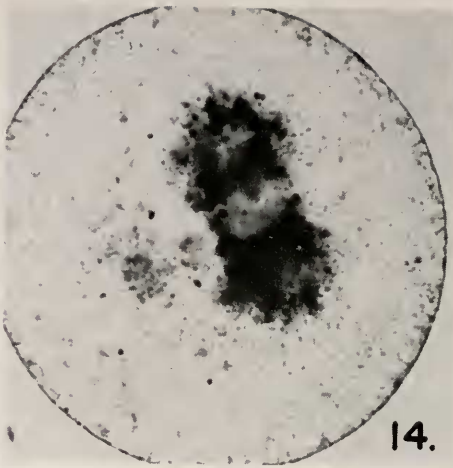
e. *Formation of the first cleavage spindle*

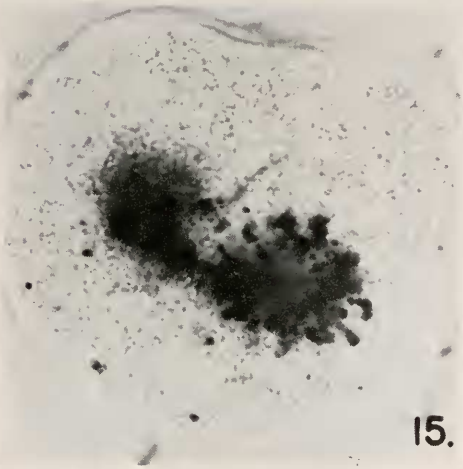
The two pronuclei are now in contact and the nuclear membranes soon break down, liberating the nuclear contents without forming a true fusion nucleus. Just prior to this, the ring of stained particles divides into two half rings when seen along the animal-vegetal axis, and the half rings soon round up into partial spheres surrounding each centrosome. (See Figures 12 and 13 for two views of the same egg, about 20 seconds apart and just before nuclear membrane breakdown.) In compressed eggs it can again be seen that the particles are excluded from a region 4 to 5 microns in diameter around the centriole.

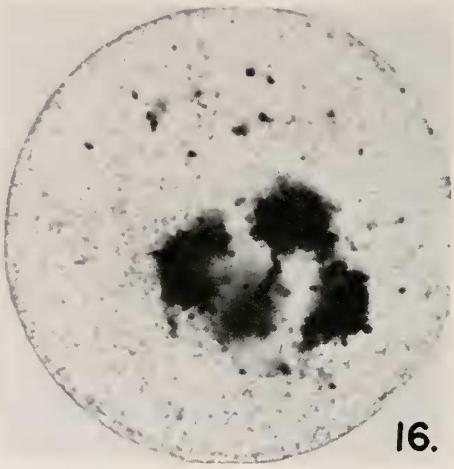
When viewed in a direction perpendicular to the animal-vegetal axis the mass of stained particles appears as a bar of material tangent to the two pronuclei (Fig. 11). This soon separates into two masses, corresponding to the division of the ring seen from the perpendicular direction.

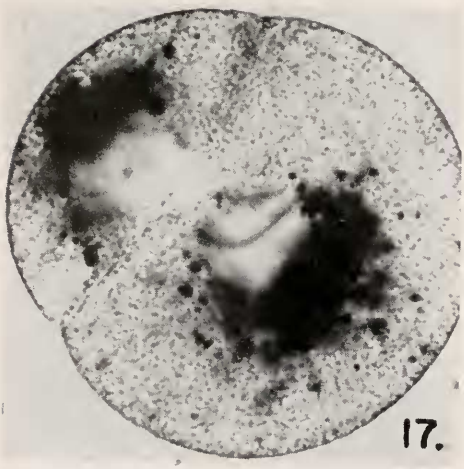
The spindle forms in the center of the egg and remains there for about 3 minutes (Fig. 14). It is then translated along its axis to the periphery of the egg

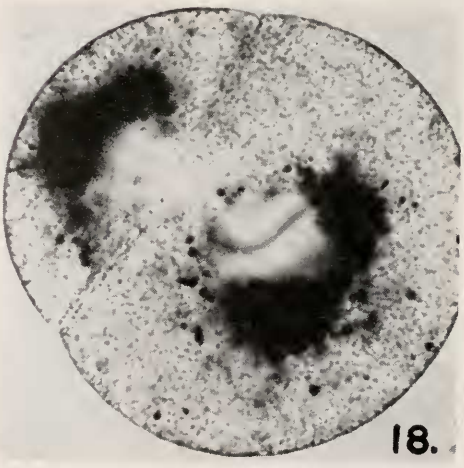












FIGURES 13–18.

just prior to cleavage. In time-lapse movies where a frame is taken each 2 seconds, this movement looks very rapid since the whole process takes only about 45 seconds in life. The spindle is now about 30 microns in length (the egg is about 60 microns in diameter) and its axis is perpendicular to the animal-vegetal axis (Fig. 15). In the majority of eggs it appears (with accurate counts not taken) that the number of stained particles about the peripheral aster is smaller than that around the central one (for a similar phenomenon in second cleavage, see Figure 23). Although the maneuvers of the stained particles in polyspermy have not yet been studied in detail, the end result has been seen many times. The stained particles appear to divide among the asters present in approximately equal numbers so that the multipolar spindles are neatly outlined at their astral apices (Fig. 16).

f. *Spindle rocking and first cleavage*

A detailed examination of the peripheral aster at this stage reveals that a cone of clear cytoplasm free of granules of any type exists from the centrosome to the egg surface (a distance of about 2 to 3 microns) (see Figure 23 for the same phenomena in second cleavage). The stained particles surround the asters, being excluded from the peripheral area just mentioned, the spindle itself and the centrosome.

Almost immediately after the spindle translates to the periphery it begins a peculiar, regular rocking motion. Although this was first seen in time-lapse movies of stained eggs, it may be seen as well in unstained eggs by visual observation. The half-period is about 30 seconds and from 4 to 8 half-periods are completed before the motion stops. The motion is one in which the central aster remains fixed and the peripheral one moves through an arc of about 30 degrees. By close observation of the clear cone between the peripheral aster and the surface, it can be seen that this clear region moves with the spindle and therefore appears to slide to and fro beneath the cell surface (Figures 20 to 22 show the rocking phenomena for second cleavage).

The movement stops just before spindle and cell elongation occurs. The cell elongates now, in a direction parallel to the spindle axis, and so forms a prolate spheroid with the spindle on its axis. Furrow formation starts at about this time and usually advances from the region of the polar bodies (animal pole) first. The plane of the furrow bisects the axis of the spindle and because of the peripheral location of the spindle, two unequal blastomeres are formed, the large CD and small AB blastomere. The stained particles outline the asters beautifully and, as

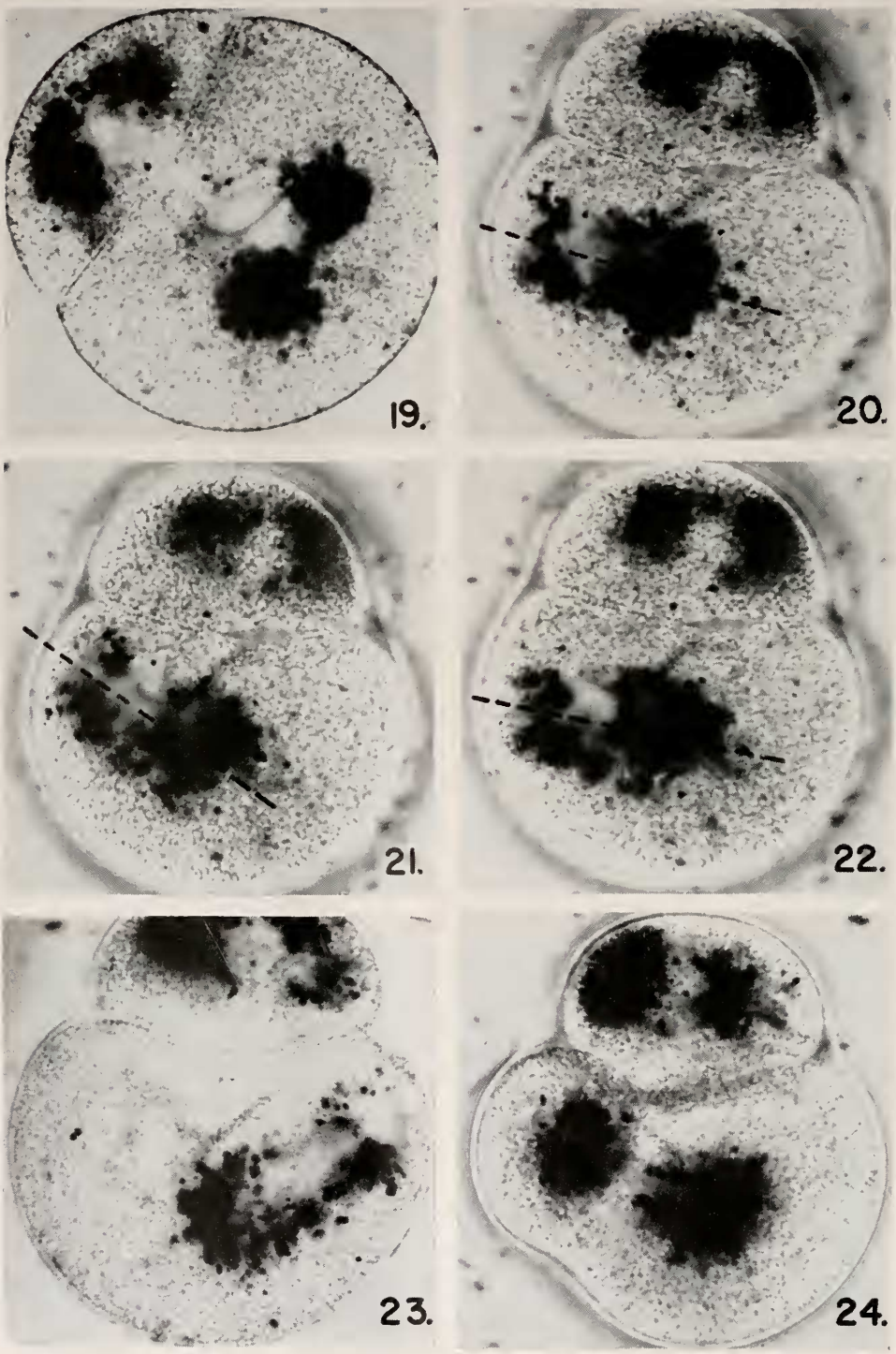
FIGURE 13. This view is of the same egg as in Figure 12, but taken approximately 20 seconds after it, and shows the beginning of the formation of the central concentration of the methylene blue stained particles.

FIGURE 14. The asters are well formed and the spindle now begins to elongate. Note the similarity of Figure 14 with Figure 4.

FIGURE 15. At about 60 minutes the first cleavage furrow starts to form, first at the animal pole. The egg has elongated in the direction parallel to the axis of the spindle. Note the radial arrangement of the clumps of particles in the aster and the "hollow" center it possesses.

FIGURE 16. Four groups of astrosomes (one slightly out of focus) indicate the ends of a tetrapolar spindle in this polyspermic egg.

FIGURES 17–18. See Figure 19.



FIGURES 19–24.

can be seen from Figure 15, almost all have localized in the asters near the centrosomes at this time.

The blastomeres round up again after the cleavage furrow has gone through. The polar bodies remain at the animal pole, beneath the vitelline membrane.

In abnormal cases arising either in eggs which have been stained by method I long enough to form compound bodies or in eggs which have been damaged by prolonged exposure to the microscope lamp, the rocking motion may continue longer (12–20 half-periods) and may attain angles up to 45°. In these cases the motion then gradually subsides with no cleavage following. On the other hand, perfectly good cleavage can occur without spindle translation to the periphery or spindle rocking. In these cases the spindle remains central in the egg and cleavage yields two equal sized blastomeres. Such cases are rare, however, and are probably anomalies in terms of later development.

In addition to the above observations, fertilized eggs previously untreated with dye were stained by method II at first cleavage. In all cases, we observed a pattern similar to that seen in eggs stained before fertilization: that is, an astral location of the metachromatic particles. In some batches of eggs, there were few, if any, discrete stained particles anywhere else in the cell. However, in most other batches of eggs, many stained particles were seen scattered throughout the cytoplasm (appearing on casual observation as a "diffuse" stain), although a heavier astral concentration always existed. The stain in the astral regions was always light compared to that which would be seen at this time in eggs which had been stained before or just after fertilization, so that eggs often had to be compressed to see astral localization.

The further maneuvers of particles stained at this late date are identical in nature to those stained before fertilization.

g. *Interphase and prophase of second cleavage division*

Nuclear reconstitution takes place by the formation and fusion of small karyomeres ("chromosomal vesicles") as can be seen most clearly from electron micrographs. This same process occurs in *Chaetopterus*, *Arbacia* (Gross *et al.*, 1958) and *Fundulus* (Richards, 1917) and is probably of quite general occurrence in molluscs (Raven, 1958). The stained particles begin to gather at one pole of the blastomere nucleus, primarily that facing the periphery, although sometimes a little to one side. The particles are rarely found on the surface of the central hemisphere of the blastomere nucleus (Fig. 17). After formation of the cap of stained particles, one sees remarkable movements in some of them in time-lapse

FIGURE 19. Figures 17 to 19 are taken of the same egg about 20 seconds apart and indicate (a) the peripheral location of the astrosomes on the blastomere nuclei and (b) the separation of the particles into two groups just prior to nuclear membrane breakdown. Unfortunately, a piece of lint traverses the image of the nucleus in the CD blastomere.

FIGURES 20–22. Microphotographs of the same egg taken about 20 seconds apart. This shows the 30° excursion of the spindle in a half rotation. Note the fixed central aster.

FIGURE 23. The oscillation in this egg has stopped and the pre-cleavage elongation has occurred. Note the peripheral cone of clear cytoplasm, the radial arrangement of the astrosomes and the smaller number of particles about the peripheral aster.

FIGURE 24. The cleavage furrow is practically completed at about 75 minutes in the CD blastomere. The AB blastomere lags by about 1 minute.

movies or by direct visual use of the light microscope. They may move very rapidly (1 to 2 microns per second) in a radial direction, shuttling back and forth in straight lines between the nucleus and the periphery. In time-lapse movies they may appear to jump discontinuously.

This movement subsides before the next division, which occurs 15 minutes after first cleavage. The prelude to this cleavage is a division of the mass of stained particles into two masses which then appear to slide over the intact nuclear surface, ultimately coming to lie at opposite poles of the nucleus (Figures 17 to 19 show this process in one egg: the pictures are taken 20 seconds apart). This undoubtedly corresponds to prophase of the second division, but the nuclear events cannot be seen easily enough to identify early stages by chromosome morphology. The division of the mass of stained particles into two sub-masses occurs about 1 minute earlier in the large CD blastomere than in the small AB blastomere, which correlates with the 1-minute difference in cleavage times (Allen, 1953).

The particles forming the two masses are arranged so as to indicate the poles of the second cleavage spindle. It can then be seen that the axis of this spindle in each blastomere makes an angle of 90° with that of the spindle of first cleavage. On the other hand, the axes of the spindles of the two blastomeres in some, but definitely not all eggs, are tipped at about 30° to each other as can be seen when looking along a line parallel to the axis of the first cleavage spindle (see Conklin, 1902, for a similar observation in *Crepidula*).

h. *Second cleavage*

Second cleavage in the AB blastomere is easily described and presents no unusual aspects. The two groups of stained particles outline the asters and show the same characteristics described for first cleavage asters, *i.e.*, aggregation of particles into the aster, movement of particles in a direction radial to the astral center, etc. (Fig. 19).

The spindle for the CD blastomere forms approximately in the center of the blastomere. It then shows a sudden movement to the periphery and toward the AB blastomere (which has not yet divided). The movement takes about 45 seconds and results in the spindle axis, in its new position, making an angle of about 30° with the axis of the spindle in the position in which it first formed. Except for this tipping, the motion is reminiscent of the spindle translation seen in first cleavage. The resemblance is further strengthened by the occurrence of a rocking motion on the part of this spindle of about the same period, amplitude, and form as in first cleavage (Figures 20 to 22 show a period in the rocking motion, the photographs having been taken about 20 seconds apart). By the same form is meant the fact that it is the peripheral aster which moves, the whole spindle rocking about the fixed central aster. Again in conformity with the events in first cleavage, a cone of clear cytoplasm exists between the peripheral aster and the egg surface, and the peripheral aster with its cone appears to slide to and fro under the surface (Fig. 23). Finally, the number of stained particles near the peripheral aster is smaller than that near the central aster (Fig. 23). It is thus clear that cleavage of the CD blastomere is very similar in course to that of the whole egg in first cleavage.

The rocking motion stops after 4 to 8 half-periods and in its final position the

spindle is tipped at about 30° to the animal-vegetal axis, that is, it has returned to the position from which it started its rocking motion (Fig. 23). The cell elongates approximately parallel to the spindle and the cleavage furrow then begins to pinch in, as in first cleavage, from the animal pole first. The result is a large D blastomere and a small C blastomere (Fig. 24).

As in first cleavage, in some cases the spindle fails to migrate to the periphery. The resulting blastomeres are then of equal size. In such cases irregular rocking motions of the spindle sometimes occur.

Later cleavages have not been studied in detail but the same process of division of the stained particles into two masses, outlining of the asters and, finally, reconstitution of a single mass on the nuclear surface has been followed as far as fifth cleavage and there is no reason to suppose it stops here. In addition, in two sequences of a time-lapse movie of third cleavage, the spindle of the D blastomere (but not that of the others) was seen to form in the center of the cell, move to the periphery, and show the same spindle rocking movements as seen in the whole egg in first cleavage and the CD blastomere in second cleavage.

It should be pointed out that the number of particles stained appears to remain the same from the unfertilized egg on, so that they appear to be diluted in each blastomere in each division. This by no means implies that new particles do not arise in each division but simply that if such particles arise it is probably not through division of previously stained ones.

i. *Centrifuged eggs*

Staining experiments with centrifuged eggs were carried out primarily with toluidine blue, although neutral red (Kojima, 1959a) was used in some runs, the results being essentially identical. The eggs were generally stratified at 8000 g for periods of 1½ to 4 minutes depending upon the batch of eggs (clams from different populations behave quite differently with respect to stratification times). In addition, a limited number of eggs were partially stratified at 4380 g for 1 minute at approximately the first polar body stage and at first cleavage.

Four types of experiments were run: (1) eggs were stained before fertilization and then stratified at various periods through second cleavage; (2) eggs were fertilized, stained for several minutes starting at 5 minutes after fertilization and then stratified at various periods through second cleavage; (3) eggs were fertilized and then stained for from 2 to 5 minutes immediately before centrifugation at various times through second cleavage; (4) eggs were fertilized, stratified at various times through second cleavage and then stained, usually for not more than 3 minutes, to avoid major redistribution of particles before observation.

Before describing the results of the above experiments, several considerations must be mentioned. First, the stratified egg begins to redistribute quickly, so that observations must be rapid. Thus, large numbers of eggs must be surveyed under low power and estimations of the location of stain in "typical" cases made in a matter of minutes. Cases may then be selected for longer study at high power (oil immersion). Second, eggs destain rapidly if oxygen is excluded from the preparation as is inevitable when compressed eggs are used. Third, in many cases the vitelline membrane and jelly coat stain heavily with dye (metachromatically) so that it is very difficult to decide whether a "diffuse" stain is, indeed,

intra- or extracytoplasmic. Fourth, the cortical granules stain blue and interfere with observations in the centrifugal portions of eggs (see below). Fifth, many refractive yolk granules in the centrifugal parts of stratified eggs place considerable restrictions on accurate observation in this zone. In assessing the results of the experiments, the reader must bear the above considerations in mind.

After germinal vesicle breakdown, the unstained, stratified *Spisula* egg shows five constant layers, with some overlap in the more centrifugal ones: most centripetally, a cap of lipid granules; next, a clear layer; then, a mitochondrial layer; a yolk layer; and, finally, most centrifugally, a layer of cortical granules (these do not break down on fertilization in the *Spisula* egg). In addition, pronuclei may be found immediately below the lipid cap after second polar body formation, and a spindle after syngamy, although the latter is by no means easy to see.

1) *Behavior of particles in eggs with a germinal vesicle (unfertilized eggs to eggs about 10 minutes after fertilization.)* The results of experiments on such eggs are the same whether staining is carried out after or before centrifugation. In all cases the majority of the metachromatic particles can be seen in a narrow layer centrifugal to the germinal vesicle (a layer identified as a mitochondrial layer with the electron microscope) (see Figure 1). Usually, a small number of the particles can be seen in the lipid cap and in the centrifugal yolk area. In about 2 to 3 per cent of the cases, many of the cortical granules accumulate at the centrifugal pole. The metachromatic particles, as seen with dye concentrations of 1 part toluidine blue in 100,000 parts sea water, are small (about ¼ to ½ micron in diameter) and stain heavily. With higher dye concentrations, many particles appear as stained vesicles more than a micron across. This is especially true with neutral red.

Some of the unfertilized eggs are parthenogenetically stimulated by the sucrose "pycnotic" barrier. In such cases particles are closely associated with the asters formed (see Figure 2).

2) *General behavior of particles in eggs after germinal breakdown.* After germinal vesicle breakdown to at least second cleavage, the metachromatic particles can be found in two locations: a layer at the centripetal end of the clear zone, and a layer at the centrifugal end of the yolk area. These general locations can be seen in all four types of staining experiments performed (*i.e.*, with relation of staining to centrifugation times), and, indeed, the localizations appear identical, although, in general, the closer the observation time is to the staining time, the *lighter* does the stain appear. The particles in both widely separated locations appear to have the same size and staining characteristics as the particles in uncentrifuged eggs (remember, however, the difficulties of observation in the yolk area). With high dye concentrations, 1 part or more toluidine blue in 50,000 parts sea water, the particles in the centripetal layer may be considerably larger than those in the centrifugal layer.

In addition, when centrifuging eggs at the stages in which polar bodies are given off or after syngamy, one sees two other phenomena in most if not all eggs; one, there is a thin, clear zone separating the centripetal layer of particles from the lipid cap (this is not present when pronuclei have formed); two, one often sees very tight knots of particles in this centripetal zone of particles. If one follows these tight aggregates for 10 to 15 minutes, one gradually sees the mass grow

larger, the radial arrangement of grape-like masses of stained particles form and, in general, the development of all the activity characteristic of asters. It would appear that the centripetal particles represent the β-particles of Pasteels (1955), by both their location in the centrifuged egg and their behavior relative to the asters. It is clear that these particles are directly stainable, since they were seen at all times in the mitotic cycle, at the most 4 minutes after the beginning of staining and, in one experiment, at 2½ minutes after the beginning of staining (see Discussion).

The results of staining centrifuged eggs at various times in the cleavage cycle differ somewhat from those with unstratified eggs so stained. If, at first cleavage, one compares an unstratified egg stained before fertilization with one stained at this stage, one finds in most batches of eggs an extensive "diffuse" stain in the latter, not found in eggs stained before fertilization, which may obscure the astral aggregation of metachromatic particles unless the eggs are compressed. In addition, the astrally aggregated particles appear to stain more lightly in eggs stained at first cleavage. The eggs stained in these two ways thus look different in gross appearance, one egg clear, the other "diffusely" stained. The centrifuged eggs look much more similar in this regard, although the eggs stained after centrifugation at first cleavage stain less heavily. In watching the latter, it can be seen that the centripetal particles (β-granules) increase in color intensity with time. This increase in intensity of β-granule staining, coupled with a destaining of the vitelline membrane and jelly coat, probably contributes to the differences in "diffuse" staining seen in the unstratified eggs.

It is clear that after germinal vesicle breakdown two groups of stained particles can be seen. We saw that this division of particles into two groups with the centrifuge occurred in eggs which had been stained before fertilization and then stratified at first cleavage. However, such eggs when observed before stratification appear to be almost completely devoid of particles anywhere in the egg except in the astral regions, and it, thus, is important to know where the two groups of stained particles come from. To get at this problem we centrifuged eggs lightly at 4380 g for 1 minute at the first polar body stage and at first cleavage. This partially stratifies the egg (a definite hyaline zone is not present, although lipid, yolk, and cortical granule layers are). In eggs stained before fertilization, the spindles with attendant stained particles were very clear. In many such eggs no metachromatic particles could be seen in the centrifugal yolk area, although this was not universally true. If eggs were stratified first and then stained, the particles in the asters were seen but were lighter in stain than in the previous case. In addition, particles were seen in the centrifugal zone, but it was impossible to determine if there were more or fewer than in the first experiment. In eggs from these same batches, centrifuged at the higher speeds and longer times discussed earlier, the two groups of particles, centripetal and centrifugal, were clearly seen.

j. *Other staining methods*

Our results with both the Nadi reaction and Janus Green B staining can be simply stated: at all stages in mitosis the stain appears only in the mitochondrial layer in centrifuged eggs, and in non-centrifuged eggs we find only a uniform distribution of stain in the cytoplasm external to the spindle (for a different result,

see Dalcq *et al.,* 1956). This result agrees with that which would be predicted from our electron micrographs of eggs at various mitotic stages, since in these micrographs, no specific localization of mitochondria can be seen in uncentrifuged eggs at any stage of mitosis. In centrifuged eggs, the electron microscope shows mitochondria to be gathered primarily into a layer just centripetal to the yolk (both in fertilized and unfertilized eggs), although they can occasionally be seen in the lipid and yolk layers (see also Pasteels *et al.,* 1958).

Discussion

The events of early cleavage in *Spisula,* which we have set forth above, are similar in many details to those described in other lamellibranch eggs such as *Unio* (Lillie, 1901), *Mactra* (Kostanecki, 1904), and *Barnea* (Pasteels, 1930). A discussion of all aspects of the cytology of these stages (which can be found in Raven, 1958) would be out of place in the present paper and we shall concentrate only on some of the more puzzling problems which these observations raise.

A problem which fits the latter category is that of spindle translation and oscillation in first cleavage, first reported by Lillie (1901) in the fresh-water clam, *Unio.* Lillie found no morphological structure revealed by his technique which could account for the precision and movement which the first cleavage spindle possesses. He felt, however, that there must be some invisible (but by no means mystical) organization of the cytoplasm which imposes its influence upon the spindle, and that this is part of that more general organization which determines the mosaic character of the egg. That he observed the spindle oscillation as well as translation seems clear from his statement that "the position of the spindle is controlled through the cytoplasm as a needle in a magnetic field oscillates until equilibrium is attained" (Lillie, 1901 : page 255).

Spindle movements in the gastropod, *Crepidula,* were described by Conklin (1902, 1912) and were ascribed by him to the influence of cytoplasmic currents. The peripheral migration of the first polar body spindle was thought to be due to an axially directed vegetal-to-animal current which moved the spindle to the animal pole. The characteristic alternation of spindle directions in third and later cleavages in *Crepidula* (and presumably in other *Spiralia*) was considered to be due to currents which themselves altered direction with cleavage in late anaphase. Experimental interference with those currents appeared to stop cleavage (Conklin, 1938).

A cortical influence on spindle displacements appears from the work of Pasteels (1930, 1931) on *Barnea candida,* and, indeed, Raven (1958) feels that the primary influence originates in the cortex. This influence presumably affects the spindle through the cytoplasm, possibly through an influence on cytoplasmic currents.

Our own observations on spindle movements, both visually (with the microscope) and in time-lapse movies, make it clear that cytoplasmic displacements and spindle movements are correlated events. However, the dissection of cause from effect in these phenomena is by no means obvious. Indeed, in many time-lapse movie sequences of spindle movement, the rapidity of its inception and apparent directedness of its trajectory leave a definite impression of an object pulled, rather than pushed, through the cytoplasm. It is not impossible that a morphological

connection of a temporary nature may attach the spindle to the egg cortex, and that contraction of this connection is the event which displaces the spindle toward the cortex.

Some indirect evidence for such a view can be cited. First, the egg cytoplasm is capable of rapid localized contractions. These can be seen in *Spisula* eggs which become amoeboid after membrane removal with alkaline isotonic NaCl (similar amoeboid phenomena in eggs have been reported by Lillie, 1902, and Monroy, 1948, in annelids; by Pasteels, 1930, and Kostanecki, 1904, in molluscs; and by Harvey, 1938, and Moser, 1940, in echinoderms). Very rapid "twitches" may occur in some parts of the eggs while other parts appear quite fluid (*i.e.*, are flowing). Second, a close relation appears to exist between the cortical granules and the spindle ends. This is a normal phenomenon in later (*i.e.*, third and on) cleavages and will be reported in detail in a later publication. In addition, in eggs treated with alkaline isotonic NaCl, as early as first cleavage cortical granules (as distinguished by size, shape and staining properties) may appear tightly aggregated about a single pole of the spindle (which in these cases does not migrate) (for such a relation in *Mactra,* see Kostanecki, 1904), indicating connections of the spindle pole with the cortex which allows the granules to be pulled to the pole under the experimental conditions described above. Such connections (if they exist) need be no more permanent than astral rays (which they may indeed be). Finally, if such connections consisted of local gelated regions of cytoplasm (fibers, rods), their movements relative to the surrounding cytoplasm might, indeed, have been described as cytoplasmic currents. All in all, one need not be bound to the idea of a fluid current as being the motive force in spindle movement.

Existence of particles

In any study using the techniques here discussed a primary question is the existence of the particles prior to treatment. The controversies concerning the origin and existence of the "vacuome" (Hovasse, 1956) visualized after neutral red staining make any such staining techniques guilty, until proven innocent, of the production of the particles by the techniques themselves. This is the more so, since a type of reaction to injury in the egg is the formation of vacuoles (Heilbrunn, 1956).

We have briefly discussed the vital staining experiments of Pasteels, Mulnard, and Dalcq in the introduction. In addition to this work with the living cell, these authors used the Gomori acid phosphatase technique and the Alcian blue technique for acid mucopolysaccharides, and were able to show, in several invertebrates, that acid phosphatase and acid mucopolysaccharides in the egg showed the same localization changes as those undergone by the dye particles in the vitally stained egg (Pasteels and Mulnard, 1957; Pasteels, 1958; Mulnard, 1958). We have confirmed some of these observations with *Spisula* and will report them at a later date. These histochemical results indicate that, at least, there is a moiety (or "plasm") of the egg which predates any treatment with dye, and which follows the centrosomes, although it does not prove that this moiety is particulate. We will discuss this problem after first comparing our results with those of the Belgian school.

Comparison of the present work with that of Pasteels, Mulnard, and Dalcq

We have already indicated that the above authors feel that there exist in the egg two types of particles: a small, lightly staining metachromatic one, called the α-granule (or α-mitochondrion) which can be present and formed at any time in the cleavage cycle, and a larger, deeply staining one which appears in the cell only at certain specific periods in the "life" cycle of the egg (although it may exist before it can be stained). In the molluscs, *Barnea* and *Gryphea* (Pasteels and Mulnard, 1957), the sea urchins, *Psammechinus miliaris* (Pasteels, 1955) and *Paracentrotus lividus* (Pasteels, 1958), and the ascidian, *Ascidiella aspersa* (Dalcq *et al.*, 1956; Dalcq, 1957), this period is primarily that during which copulation of the pronuclei is beginning to occur, although some particles may be formed at the time of the second maturation division. In *Chaetopterus pergamentaceus* (Mulnard, 1958) the particles begin to appear at the first maturation division.

Several bits of evidence are adduced by the Belgian workers for the existence of two particles, the most telling being the so-called indirect staining of the β-granules. That is, the observation that eggs stained at cleavage show only a diffuse light granular stain (α-particles), the astral location only occurring at the next cleavage and then in particles larger and more darkly staining than the first. In *Chaetopterus*, however (Mulnard, 1958), there appears to be some question as to whether the β-particles are not directly stainable since in several cases Mulnard reports their appearance after staining "à un stade où les granules β existent déjà en grande nombre" (Mulnard, 1958, page 657), and, therefore, their direct stainability. He feels, however, that this is probably due to their rapid formation during the five-minute staining period he used and during which time material may be transferred to the β-granules from α-granules.

Two further bits of supporting evidence for the two-particle idea come from vital staining experiments and from histochemical tests. Thus, Pasteels (1958) claims that in *Paracentrotus* (the one exceptional species, so far) the α-granules do not stain with the acid phosphatase technique (although the β-granules do). In addition, the α-granules stain with neutral red but the β-granules do not, and thus neutral red particles do not show an astral location. This last result should, however, be contrasted to the neutral red results obtained by Iida (1942) in a Japanese species of sea urchin (species not given) and by Kojima (1959a) in three species of sea urchins.

It is clear from the above discussion that the same basic phenomena have been described by ourselves and the Belgian group. It is equally clear, however, that differences exist. In view of the variation in results which that group have themselves reported in different species, it can be assumed that at least a part of our differing observations can be traced to different materials.

Our own vital staining work supports the idea that the particles pre-exist the treatment with dye. Thus, in all cases staining of eggs immediately before or immediately after centrifugation at any point in the cleavage cycle gives essentially the same result (keeping in mind the precautions noted in the section on observations). Indeed, if the dye were causing the formation of particles (or vacuoles) from a pre-existing, but non-particulate, moiety of the egg, one would expect the vacuoles to have a different relative specific gravity from this "precursor" and, therefore, to show a different centrifugal behavior from it, which the experiments

discussed in Observations, section II i, show to be false. This change in relative specific gravity is, however, what occurs in the eggs heavily stained with methylene blue, where the particles are deliberately caused to swell, and where they centrifuge to the upper stratum of the yolk layer.

A question of great interest is that of the number of types of stained particles present. Our centrifugation experiments described earlier indicate, in agreement with the work of the Belgian group, that after germinal vesicle breakdown, two groups of particles are present, one group centrifuging to the centripetal part of the hyaline layer (for more detail, see observations) and one, to the centrifugal end of the yolk layer. These particles appear to be about the same size (they are too small for very accurate measurement) and to stain with approximately the same intensity if examined 10 to 15 minutes after staining. The location of the particles in the stratified egg seems to agree with the locations of the α- and β-granules of the Belgian group. The fact that the centripetal particles migrate into the asters in redistributing eggs leaves little doubt of this identification. We may, therefore, identify our centripetal particles with the β-granules of the Belgian group, and the centrifugal particles with their α-granules. With this identification it is clear that a fundamental difference of observation exists between the work reported here and that of the Belgian school, namely, that we find the β-granules to be stainable directly and to be present and identifiable at all points in the cleavage cycle after germinal vesicle breakdown. This is supported by direct observation on compressed eggs stained with low dye concentrations at appropriate times in the cleavage cycle, although, as was mentioned in the section on observations, the difficulties due to "diffuse" staining must be carefully overcome. In addition, the observable fact that β-granules increase in depth of stain with time must be taken into account.

A further difference between our results and those of the Belgian group (although one fraught with some observational difficulties as previously discussed) derives from the results of centrifuging eggs at first cleavage, which were stained before fertilization (or soon after). Eggs at first cleavage show essentially no stained particles elsewhere in the egg other than in the asters. In lightly centrifuged eggs one finds many cases in which metachromatic particles are found only in the (centripetally located) asters. However, such eggs stratified with the higher forces show the two groups of particles, α- and β-granules. It would appear, therefore, that both α- and β-granules, in *Spisula*, show an astral aggregation, which is maintained under low, but disrupted under high centrifugal forces.

To reiterate, then, in *Spisula* eggs one finds two sets of particles, both directly stainable with toluidine blue (or neutral red, etc.) and both of which show the migration into the asters. What the relationship between these particles is (and to what degree the centrifugal behavior of the particles represents a real difference in their nature) we cannot say from our observations. Our work neither lends support nor in any way detracts from the idea of the Belgian groups that the α-granules are precursors to the β-granules. However, in *Spisula,* if this is so, the process of β-granule formation must be continuous throughout the cleavage cycle, with the β-granules so formed, directly stainable.

Direct confirmation of the existence of the particles in the untreated living cell would still be highly desirable. Dalcq *et al.* (1956) report that particles

showing the behavior under discussion can be seen, with the phase microscope, in the living egg. However, using both phase and interference microscopy, with and without the immersion media earlier discussed, we have not been able to confirm this observation, although it is true that occasionally dark particles (in dark contrast phase), or particles of a slightly different hue from other particles in the cell (in interference microscopy), appear near the centrosomes in living, unstained eggs. Unfortunately, the presence of many highly refractile granules in eggs makes such observations unreliable, since even in compressed eggs halos make detailed observations difficult in phase microscopy. Reversed color effects from particles not in the image plane make interference microscope images also unreliable in this case. In addition, the contrast in a phase microscope is a function of the optical path difference between an object and its surround (Bennett *et al.*, 1951), and, since the average refractive index of the cytoplasm outside the asters and spindle is different from that of the asters and spindle themselves (Ross and Rebhun, unpublished experiments; Mitchison and Swann, 1953), it would be expected that particles near the asters would show some contrast difference with those elsewhere. These disappointing results may simply mean that the optical path difference between these particles and the cytoplasm and that between other cell particles and the cytoplasm is about the same and the particles are therefore not distinguishable by this property alone.

A similar disappointing result has so far been obtained by us with the electron microscope. Our work (Rebhun, 1958) and that of Pasteels *et al.* (1958) seem definitely to remove mitochondria from consideration as candidates for the stained particles, reversing a previously stated suggestion (Pasteels and Mulnard, 1957; Dalcq *et al.*, 1956).[3] Pasteels *et al.* (1958) feel that in centrifuged eggs of the sea urchin, *Paracentrotus lividus*, they can see "Golgi" bodies in the layer to which the acid phosphatase positive, metachromatic β-granules go. Unfortunately, they present no electron micrographs to support this claim. In addition, they do not see these bodies specifically associated with the asters. In our own electron micrographs of *Spisula* we can occasionally see at least two different types of particles possibly associated with the asters, one of which looks superficially like Golgi bodies in having closely packed concentric lamellae surrounding a vacuole. However, this may merely indicate a complex lipid cortex such as occurs in the methylene blue, brilliant cresyl blue and Nile blue staining phospholipid particles in pulmonate snail neurons (Chou, 1957; Ross and Chou, 1957). Indeed, in electron micrographs of such neurons, bodies, such as described above in electron micrographs of *Spisula* eggs, do occur (Ross, personal communication). The second

[3] I would like to clear up a misunderstanding concerning these results which apparently occurred during a conversation with Dr. Mulnard and which I discovered only upon reading his paper (1958). My position concerning the existence of the particles is there reported as one of scepticism which, it is claimed, stems from electron micrographs of heavily stained eggs (method I). These micrographs do, in fact, show vesicular bodies not seen in unstained eggs, with single or double membranes outside and vesicles or "cristae"-like (Palade, 1953) objects within. It was clear to me, however, as discussed in this paper, that these large bodies arise from the prolonged action of dye upon a pre-existing substratum. Among the possible particles which might be involved as substratum, I considered at that time, a particle with the morphology of mitochondria but with different enzymatic properties, but realized this to be very hypothetical. I now reject this possibility. I have never thought these particles to be degenerated, "ordinary" mitochondria as stated by Mulnard (1958).

type of particle resembles the multivesicular bodies in rat eggs (Sotelo and Porter, 1959). All in all, the electron microscope is disappointingly vague on the questions relevant here. We shall, however, report these results in detail at a later date.

Some observations which may have a bearing on the problem of the general existence of the particles other cells, are those of Bloom *et al.* (1955, Fig. 2) on newt heart fibroblasts. The movies from which this paper is taken show many dark particles (called "fat droplets" by the authors) which migrate to the centriole region of the cell in prophase and show a distribution in division very similar to that described above in eggs. Indeed, there appears to be enhanced movement of many of the particles and an aggregation into grape-like masses during division, similar to that described in eggs. Histochemical studies of these cells, however, have not been reported.

Finally, Holt (1957) showed that esterase and acid phosphatase in mitoses, after formalin fixation, in regenerating rat liver showed the same localization changes that the basic dyes and acid phosphatase do in eggs.

Preliminary identification of the particles

Work has already been quoted which indicates that these particles are not mitochondrial in nature. We have also indicated our attitude of constraint (not, however, negation) toward the suggestion that the β-particles are themselves Golgi bodies. The suggestion remains (see Mulnard, 1958) that the α-granules are lysosomes in the terminology of de Duve (1957), this being supported by the fact (ignoring Pasteels' (1958) negative result in *Paracentrotus* for α-granules, for the moment) that the α-granules contain acid phosphatase. The lysosome suggestion is strengthened by the observation of Holt (1957) that acid phosphatase in liver parenchymal cell mitosis shows the same behavior as our particles do in living eggs, since the lysosome concept originated from particles obtained from liver. Our preliminary work with egg homogenates indicates that the acid phosphatase of the egg is sedimentable at relatively low speeds, rather than soluble. However, we find no evidence that the activity is increased by treatment with Triton-X, aging at 37° C., freezing and thawing, or treatment with distilled water. Thus, the egg particles lack a fundamental property of lysosomes, *i.e.*, a *releasable* acid phosphatase. These results will be reported in detail at a later date.

Motion of the particles

As has been pointed out, the particles may move with great rapidity and in fixed directions. This motion is superimposed on a Brownian motion similar to that undergone by other particles in the cytoplasm. The characteristics of the motion of the stained particles are: (a) they may move with velocities up to several microns per second; (b) two particles within a few microns of each other may move rapidly in opposite directions; (c) the rapid movements are, generally, radial to the centrosome, either towards or away; (d) not all particles move rapidly at any given time, *i.e.*, some may be undergoing ordinary Brownian motion; and (e) these motions are *not* participated in by most other inclusions in the cell (the motions of cortical granules in later cleavages may be an exception). Any mechanism proposed to explain these phenomena must explain them all.

There are several types of forces which might be invoked to account for these events. For example, Bjerknes (see Schrader, 1953) published accounts of differential movement of particles relative to each other on fluids in which standing waves were set up by two pulsating spheres. The movement of the particles towards or away from the spheres depended on the phase of the vibrating spheres and the density of the particles relative to the fluid medium. This phenomenon has been used in models of anaphase separation of chromosomes (Schrader, 1953), and Pfeiffer (1956) has published accounts of supposedly pulsating centrosomes. We feel, however, that much more evidence for such standing waves or pulsating centrosomes would have to be gathered before such a mechanism could be considered as causing particle movement in eggs. In addition, the α- and β-granules appear to participate in the movement although their centrifugal behaviors, and therefore densities, are very different.

The intermittent growth of the astral fibers was postulated by Iida (1942) as causing the motion of the particles. This requires the particles to be attached to the astral rays in some way. Their normal Brownian motion, however, would seem to argue against such an attachment (although the fact that they centrifuge with the asters might support it). Also, the net direction of motion of the particles during mitosis is into the asters at a time when the asters are growing and, therefore, moving out. Finally, there is no evidence of a shortening of astral rays during their growth period as is required to pull the particles into the asters on the above model.

Cytoplasmic currents might be invoked as agents sweeping the particles into the asters. Conklin (1902) and Chambers (1917) considered the aster to be essentially composed of such streams. We feel, however, that certain observations speak against this notion, namely, the motion of some stained particles relative to nearby stationary ones, and, more particularly, their motion relative to yolk granules and mitochondria in the same region which remain stationary (apart from Brownian movement). Currents should be relatively indiscriminate in moving small particles of approximately the same size. Moreover, the motion of some of the particles is so rapid, and starts so suddenly, that it is difficult to imagine currents being involved. Finally, the same particle may be rapidly "jerked" into the aster and just as rapidly reverse its movement, although the net direction is inward as cleavage proceeds.

In connection with the last observation, we suggest the possibility of mechanical connections of some sort, possibly contractile fibers or gel streams, connecting the stained bodies to the centers. Contractile fibers of this nature have been postulated as being involved in the motion of echinochrome granules in *Arbacia* (Parpart, 1953). After fertilization, these particles move radially into the egg cortex (McClendon, 1910). In movies of this phenomenon, it is clear that the motion of echinochrome granules has exactly the same five properties as listed for the metachromatically stained particles, if we replace the word "centrosome" by the words "egg cortex" in (c) above (A. K. Parpart, personal communication).

We suggest, again, as for the case of spindle movement, that contracting gel streams, similar to those described by Allen (1955) in amoebae, might very well be involved in these motions, and that such streams might easily be described as cytoplasmic currents.

A detailed study of the relative movements of individual particles of different types at different times in the mitotic cycle, similar to the study of Hiramoto (1958) on cleaving *Clypeaster* eggs, would be most useful in deciding between some of the suggestions discussed above.

Function of the particles

Some recent work has thrown interesting light on some possible functions the particles may perform. Marsland (1958) has extensive evidence from pressure centrifugation work on premature furrowing in *Arbacia* eggs that a factor from the nucleus and one from the metachromatic particles are involved in the initiation of the cleavage furrow. This work is supported by the beautiful work of Kojima (1959a, 1959b) which indicates that halves of eggs obtained by splitting with the centrifuge, cleave or do not cleave depending upon whether they do or do not receive, during centrifugation, particles such as we have been discussing. In the sea urchins *Temnopleurus* and *Mespilia* stratified but not broken eggs contain particles in the centrifugal end and it is this end which cleaves subsequently. Other evidence was adduced by Kojima in support of the hypothesis that the particles are involved in cleavage initiation.

Summary

1. Techniques for vitally staining oocytes of *Spisula solidissima* with certain basic dyes are described.

2. Such staining reveals small particles in the cytoplasm of the egg.

3. After fertilization the particles show very specific movements and localization changes which can be described by saying that the particles follow the centrioles through cleavage.

4. Spindle movements and position changes can easily be followed and are described in detail.

5. The results are discussed in the light of related work in the literature.

LITERATURE CITED

Allen, R. D., 1953. Fertilization and artificial activation in the egg of the surf-clam, *Spisula solidissima*. *Biol. Bull.*, **105**: 213–239.

Allen, R. D., 1955. Protoplasmic streaming in amoeba. *Biol. Bull.*, **109**: 339–340.

Bennett, A., H. Osterberg, H. Jupnik and O. Richards, 1951. Phase Microscopy. Principles and Applications. John Wiley and Sons, Inc., New York.

Bloom, W., R. Zirkle and R. Uretz, 1955. Irradiation of parts of individual cells. II. Effects of chromosomal and extra-chromosomal irradiation on chromosome movements. *Ann. N. Y. Acad. Sci.*, **59**: 503–513.

Chou, J. T. Y., 1957. The cytoplasmic inclusions of the neurones of *Helix aspersa* and *Limnea stagnalis*. *Quart. J. Micr. Sci.*, **98**: 47–58.

Chambers, R., 1917. Microdissection studies. II. The cell asters, a reversible gelation phenomenon. *J. Exp. Zool.*, **23**: 483–506.

Conklin, E. G., 1902. Karyokinesis and cytokinesis in the maturation, fertilization and cleavage of *Crepidula* and other Gasteropoda. *J. Acad. Nat. Sci. Phila.*, **12**: 1–121.

Conklin, E. G., 1912. Experimental studies on nuclear and cell division in eggs of *Crepidula*. *J. Acad. Sci. Phila.*, **15**: 503–591.

Conklin, E. G., 1938. Disorientations of development in *Crepidula plana* produced by low temperatures. *Proc. Amer. Philos. Soc.*, **79**: 179–212.

DALCQ, A., 1954. Fonctions cellulaires et cytochimie structurale dans l'oeuf de quelques Rongeurs. *C. R. Soc. Biol.*, **148**: 1332–1373.

DALCQ, A., 1957. La métachromasie *in vivo* au Bleu de Toluidine dans l'oeuf et l'embryon d'Ascidie. *Bull. Soc. Zool France*, **82**: 296–316.

DALCQ, A., J. J. PASTEELS AND J. MULNARD, 1956. Recherches sur la métachromasie *in vivo* au Bleu de Toluidine chez quelque Invertébrés marins. *Bull. l'Acad. Roy. de Belge. Classe de Sci., Ser. V*, **42**: 771–777.

DE DUVE, C., 1957. The enzymatic heterogeneity of cell fractions isolated by differential centrifugation. *Symp. Soc. Exp. Biol.*, **10**: 50–61.

GROSS, P., D. E. PHILPOTT AND S. NASS, 1958. The fine structure of the mitotic spindle in sea urchin eggs. *J. Ultrastructure Res.*, **2**: 55–72.

HARVEY, E. B., 1938. Parthenogenetic merogony or development without nuclei of the eggs of sea urchins from Naples. *Biol. Bull.*, **75**: 170–188.

HEILBRUNN, L. V., 1956. Dynamics of Living Protoplasm. Academic Press, Inc., New York.

HIRAMOTO, Y., 1958. A quantitative description of protoplasmic movement during cleavage in the sea-urchin egg. *J. Exp. Biol.*, **35**: 407–424.

HOLT, S. J., 1957. Changes in enzyme distribution during the mitotic cycle. Presented at 9th Internat. Congr. of Cell Biology, St. Andrews, Scotland.

HOVASSE, R., 1956. Le vacuome animal. *In:* Protoplasmatalogia, Bd. III. D#2.

IIDA, T. T., 1942. An observation on the nature of the asters. *Zool. Mag.* (*Tokyo*) (*Dobutsugatsu Zassi*), **54**: 364–366.

KOJIMA, M. K., 1959a. Relation between the vitally stained granules and cleavage-activity in the sea urchin egg. *Embryologia*, **4**: 191–209.

KOJIMA, M. K., 1959b. On the vitally stainable granules in the egg of the echiuroid, *Urechis unicinctus*. *Embryologia*, **4**: 211–218.

KOPAC, M., 1955. Cytochemical micrurgy. *Internat. Rev. Cyt.*, **4**: 1–30.

KOSTANECKI, K., 1904. Cytologische Studien an künstlich parthenogenetisch sich entwickelnden Eiern von *Mactra*. *Arch. für Mikr. Anat. Entwickl.*, **64**: 1–98.

LILLIE, F. R., 1901. The organization of the egg of *Unio*, based on a study of its maturation, fertilization and cleavage. *J. Morph.*, **17**: 227–286.

LILLIE, F. R., 1902. Differentiation without cleavage in the egg of the annelid *Chaetopterus pergamentaceus*. *Arch. f. Entw.*, **14**: 477–499.

MARSLAND, D., 1958. Nuclear and cytoplasmic factors in cleavage induction. *Anat. Rec.*, **132**: 473.

MCCLENDON, J. F., 1910. On the dynamics of cell division. II. Changes in permeability of developing eggs to electrolytes. *Amer. J. Physiol.*, **27**: 240–275.

MITCHISON, J. M., AND M. M. SWANN, 1953. Measurements on sea urchin eggs with an interference microscope. *Quart. J. Micr. Sci.*, **94**: 381–390.

MONROY, A., 1948. A preliminary approach to the physiology of fertilization in *Pomatoceros triqueter L.* *Ark. für Zool.* B40A, No. 21.

MOSER, F., 1940. Studies on a cortical layer response to stimulating agents in the *Arbacia* egg. III. Response to non-electrolytes. *Biol. Bull.*, **78**: 68–91.

MULNARD, J., 1958. La métachromasie *in vivo* et son cytochimique dans l'oeuf de l'annélide *Chactopterus pergamentaceus*. *Arch. Biol.*, **69**: 645–685.

NACHLAS, M. M., KWAN-CHUNG TSOU, E. DE SOUZA, CHAO-SHING CHEN AND A. M. SELIGMAN, 1957. Cytochemical demonstration of succinic dehyrdogenase by the use of a new p-nitrophenyl substituted ditetrazole. *J. Histochem. and Cytochem.*, **5**: 420–436.

PALADE, G., 1953. An electron microscope study of the mitochondrial structure. *J. Histochem. and Cytochem.*, **1**: 188–211.

PARPART, A. K., 1953. The motion of echinochrome granules in the unfertilized egg of *Arbacia punctulata*. *Biol. Bull.*, **105**: 368.

PASTEELS, J. J., 1930. Les effets de la rupture de la balance des chlorure de l'eau de mer sur l'oeuf de Pholade, *Barnea candida*. *Arch. Biol.*, **40**: 247–355.

PASTEELS, J. J., 1931. Recherches sur le déterminisme du mode de segmentation des Molluscque Lamellibranche. *Arch. Biol.*, **42**: 389–413.

PASTEELS, J. J., 1955. Evolution de la métachromasie au Bleu de Toluidine suivie sur le vivant, au cours de premieres phases du développement de l'oursin et de la Phalade. *Bull. l'Acad. Roy. de Belge. Classe de Sci., Ser. V.*, **41**: 761–768.

PASTEELS, J. J., 1958. Nouvelles recherches sur la métachromasie *in vivo* et sur l'histochimie de l'oeuf normal et centrifugé de *Paracentrotus lividus*. *Arch. Biol.*, **69**: 591–619.

PASTEELS, J. J., AND J. MULNARD, 1957. La métachromasie *in vivo* au Bleu de Toluidine et son analyse cytochimique dans les oeufs de *Barnea candida*, *Gryphea angulata* (lamellibranches), et de *Psammechinus miliaris*. *Arch. Biol.*, **68**: 115–163.

PASTEELS, J. J., P. CASTIAUX AND G. VANDERMEERSCHE, 1958. Ultrastructure du cytoplasme et distribution de l'acide ribonucléique dans l'oeuf fécondé, tant normal que centrifugé de *Paracentrotus lividus*. *Arch. Biol.*, **69**: 627–643.

PFEIFFER, H. H., 1956. Über die Mitwirkung rhythmischer Oscillationen der Centrosomen bei mitotischen Chromosomenbewegungen. *Protoplasma*, **46**: 585–596.

RAVEN, C. P., 1958. Morphogenesis: The Analysis of Molluscan Development. Pergamon Press, New York.

REBHUN, L. I., 1956a. Electron microscopy of basophilic structures of some invertebrate oocytes. I. Periodic lamellae and the nuclear envelope. *J. Bioph. and Bioch. Cyt.*, **2**: 93–104.

REBHUN, L. I., 1956b. Electron microscopy of basophilic structures of some invertebrate oocytes. II. Fine structure of the yolk nucleus. *J. Bioph. and Bioch. Cyt.*, **2**: 159–170.

REBHUN, L. I., 1957. Vital staining of eggs of *Spisula solidissima* by methylene blue. *Biol. Bull.*, **113**: 353.

REBHUN, L. I., 1958. Behavior of metachromatic granules during cleavage in *Spisula*. *Biol. Bull.*, **115**: 325.

RICHARDS, A., 1917. The history of the chromosomal vesicles in *Fundulus* and the theory of genetic continuity of chromosomes. *Biol. Bull.*, **32**: 249–290.

RIES, E., 1937. Die Verteilung von Vitamin C., Glutathion, Benzidin-Peroxydase, Phenolase (Indophenoblau-Oxydase) und Leuko-methylenblau-Oxydoreductase während der frühen Embryonalentwicklung verschiedener wirbelloser Tiere. *Pubbl. Staz. Zool. Napoli*, **16**: 363–401.

ROSS, K., 1954. Measurement of the refractive index of cytoplasmic inclusions in living cells by the interference microscope. *Nature*, **174**: 836–837.

ROSS, K., AND J. T. Y. CHOU, 1957. The physical nature of the lipid globules in the living neurones of *Helix aspersa* as indicated by measurements of refractive index. *Quart. J. Micr. Sci.*, **98**: 341–348.

SCHECHTER, V., 1941. Experimental studies upon the egg cells of the clam, *Mactra solidissima*, with special reference to longevity. *J. Exp. Zool.*, **86**: 461–480.

SCHRADER, F., 1953. Mitosis. Columbia University Press, New York. Second Edit.

SOTELO, J. R., AND K. R. PORTER, 1959. An electron microscope study of the rat ovum. *J. Bioph. and Bioch. Cyt.*, **5**: 327–341.

WILSON, E. B., 1925. The Cell in Development and Heredity. The Macmillan Company, New York.

WORLEY, L. E., 1944a. Studies of the supravitally stained Golgi apparatus. II. Yolk formation and pigment concentration in the mussel, *Mytilus californianus*. *J. Morph.*, **75**: 77–102.

WORLEY, L. E., 1944b. Studies of the supravitally stained Golgi apparatus. III. The methylene blue technique and some of its implications. *J. Morph.*, **75**: 261–290.

WORLEY, L. E., AND S. WORLEY, 1943. Studies of the supravitally stained Golgi apparatus. I. Its cycle in the tectibranch mollusc *Navanax inermis* (Cooper). *J. Morph.*, **73**: 365–400.